

Acrolein Depletes the Neuropeptides CGRP and Substance P in Sensory Nerves in Rat Respiratory Tract

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The mammalian respiratory tract is densely innervated by autonomic and sensory nerves around airways and blood vessels. Subsets of these nerves contain a number of putative neurotransmitter peptides, such as substance P and calcitonin gene-related peptide (CGRP) in sensory nerves and vasoactive intestinal polypeptide (VIP), possibly serving autonomic functions. CGRP is also found in endocrine cells in rat airway epithelium. These peptides are all pharmacologically potent effectors of bronchial and vascular smooth muscle and bronchial secretion. Their functions *in vivo* are less well established. We have therefore examined the effects of inhaled acrolein, a sensory irritant, on three pulmonary neuropeptides: CGRP, substance P, and VIP. Groups of rats ($n = 3$ each) were exposed for 10 min to acrolein in air (Ct = 510, 1858, and 5693 mg·min/m³) or to air alone. Fifteen minutes later they were killed (pentobarbitone IP) and their respiratory tracts were dissected and fixed in 0.4% *p*-benzoquinone solution. Cryostat sections were stained by indirect immunofluorescence for a general nerve marker (PGP 9.5) and neuropeptides. The acrolein-treated animals had a dose-related decrease in tracheal substance P- and CGRP-immunoreactive nerve fibers compared with controls. No change was seen in total nerve fiber distribution and number (PGP 9.5) or VIP immunoreactivity, nor in CGRP-immunoreactive epithelial endocrine cells. It is concluded that the rat tracheal peptidergic nerves are a sensitive indicator of inhaled irritant substances. Their reduced immunoreactivity may be because of a release of sensory neuropeptides that could play a role in the physiological response to irritant or toxic compounds.

Introduction

The respiratory tract is densely innervated by sensory and automatic fibers (1) that together with endocrine cells in the epithelium of airways form the so-called diffuse neuroendocrine system. Subsets of both the nerves and endocrine cells are known to contain bioactive regulatory peptides that can act locally as hormones or possibly as neurotransmitters/modulators (2). The neuropeptides include substance P (3) and other tachykinins (4); calcitonin gene-related peptide (CGRP), also found in endocrine cells (5); vasoactive intestinal polypeptide (VIP) (6); peptide histidine isoleucine or methionine (PHI/M) (7); galanin (8); and neuropeptide

tyrosine (NPY) (9,10). These peptides have strong actions on vascular and airway smooth muscle and glandular secretion (11,12). The distribution of nerves containing the different peptides may be analyzed by examining tissue sections immunostained with specific antisera to each peptide (2).

Of the neuropeptides in the respiratory tract, two are known to be predominantly in the sensory nervous system. These are CGRP and substance P. This sensory system is known to respond to inhaled irritants such as cigarette smoke (13), resulting in a release of neuropeptides and thus decreasing the peptide immunoreactivity of the fibers. Such local release of substance P following nerve stimulation has been observed (12,14) and shown to cause local vasodilatation and plasma extravasation (13).

Acrolein is an unsaturated aliphatic aldehyde with toxic and sensory irritating effects (15–19). It is a component of cigarette smoke (20), engine exhausts (21), and industrial by-products (22). We have, therefore, examined by immunocytochemical techniques the effect of acute exposure to acrolein vapor in the rat. The aim was to

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Table 1. Characteristics of antisera used in this study.

Antigen	Type	Carrier ^a	Cross-linker	Dilution	Absorption ^b
PGP 9.5	Whole molecule, human brain	—	—	1:1500	0.1
Rat α CGRP	Whole molecule, synthetic	BSA	Glutaraldehyde	1:200	0.5
Substance P	Whole molecule, synthetic	BSA	Glutaraldehyde	1:1000	1.0
VIP	Whole molecule, synthetic	KLH	Glutaraldehyde	1:2000	1.0

^aBSA, bovine serum albumin; KLH, keyhole limpet hemocyanin.

^bConcentration of homologous antigen (nmole/mL diluted antiserum) required to abolish immunostaining in tissues used in this study.

All peptide antisera were raised in rabbits at the Royal Postgraduate Medical School, Hammersmith Hospital, London, UK. Antiserum to PGP 9.5 was purchased from Ultraclone, Cambridge, UK.

determine whether or not acrolein could cause changes in the innervation of the respiratory tract, including the neuropeptides, and thus whether such an examination would prove to be a useful and sensitive indicator of the acute response to such irritants.

Materials and Methods

Groups of three Porton strain Wistar female rats (weight 190–210 g) were exposed for 10 min in individual wire mesh cages in a 501 (aluminum and glass) chamber, through which air was drawn at 120 L/min. Acrolein vapor concentrations were generated in the chamber by injecting liquid acrolein (BDH) at a constant rate from a syringe drive into the chamber inlet, through a 27-gauge needle surrounded by a concentric air jet. Vapor concentrations were set by adjusting the syringe drive delivery rate. Three concentrations of acrolein were used with different groups of animals, and another group (controls) was exposed to air alone.

Acrolein chamber concentrations were determined by sampling at 1 L/min into distilled water in a glass bubbler, cooled in an ice bath; the sample was diluted to a suitable volume (25, 50, or 100 mL), and the absorbance was measured in a UV spectrophotometer at 209 nm. The acrolein concentration was calculated by comparison with a standard curve.

Fifteen minutes after termination of exposure the animals were killed by pentobarbitone overdose injected IP. The thorax was opened, the respiratory tract dissected out, and specimens were taken from upper and lower trachea and lung (a 3-mm thick slice at the hilar level of right lower and left lobes). The tissues were immediately fixed by immersion for 2 hr in 0.4% solution of *p*-benzoquinone in phosphate-buffered saline (PBS) (0.01 mole/L phosphate buffer, pH 7.4, containing 0.15 mole/L NaCl), then washed and stored at 4°C in PBS containing 0.15 mole/L sucrose and 0.01% sodium azide.

Cryostat blocks were prepared and frozen sections (7 μ m thick) taken up on poly-L-lysine-coated slides. From each block, one section was stained by hematoxylin and eosin for morphological examination, and 10 sections were immunostained for each antiserum. Antisera to the general neuronal marker PGP 9.5 (23) and the neuropeptides CGRP, substance P, and VIP were used with a modified indirect immunofluorescence method (5). Briefly, slides were placed for 30 min in PBS containing 0.2% (v/v) Triton X-100, and the sections were then in-

cubated for 16 hr at 4°C with appropriately diluted primary antiserum (Table 1). Slides were washed in three baths of PBS for 10 min each and the primary antiserum was reapplied for 4 hr at room temperature. Slides were washed again (3 \times 5 min) in PBS, goat anti-rabbit IgG antibody conjugated to fluorescein isothiocyanate (Miles Laboratories), diluted 1:200, applied for 60 min, rewashed as before in PBS. Slides were mounted in PBS glycerol (1:9 v/v) and viewed using an Olympus Vanox fluorescence microscope. Photographs were taken on Kodak Tri-X film developed in Acutol. The specificity of immunostaining was verified by repeating the immunostaining but by using primary antiserum absorbed with homologous antigen (Table 1), which abolished the immunoreactivity.

The number of immunoreactive nerve fibers was assessed visually in all sections and graded from absent (–) to abundant (+++). The screening was performed randomly without knowledge of the treatment group by one observer, and 1 in 10 of the slides were checked by another observer to ensure consistency.

Results

Acrolein chamber concentrations and Ct values are given in Table 2. The low- and medium-dose animals showed no major changes in respiratory tract morphology in comparison with controls. In the medium-dose group there was slight edema and occasional small areas of bleeding into the lungs; this was more marked in the high-dose-treated animals, in which many of the airways contained cellular serous exudate. The results of immunostaining are summarized by the diagrams in Figures 1–3.

PGP 9.5

The overall innervation of all three areas of respiratory tract examined showed no change with acrolein treatment. Immunoreactive nerve fibers were seen in the

Table 2. Concentrations and doses of acrolein used.

Animal group	Concentration,	Parts per million	Ct ^a
	mg/m		mg/min/m
Controls	0	—	—
Low dose	51	22.2	510
Medium dose	185.8	81.1	1858
High dose	569.3	248.6	5693

^aCt, concentration \times time.

Nerves in upper trachea



FIGURE 1. Number of nerve fibers immunoreactive for PGP 9.5 and neuropeptides in upper trachea of acrolein-treated and control rats. The histogram bars represent the modal value in 10 sections from three animals for each dose group, the T-bars represent the range of values. The number of immunoreactive nerves is a visual estimate: 0, no nerves seen; + sparse; ++, few; +++, moderate; +++++, abundant.

Nerves in lower trachea

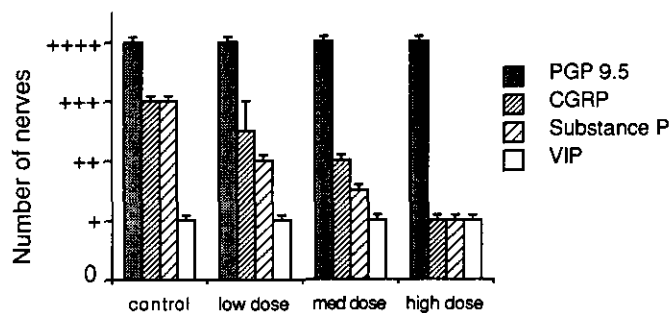


FIGURE 2. Numbers of nerve fibers immunoreactive for PGP 9.5 and neuropeptides in lower trachea, as shown in Figure 1.

Nerves in lung

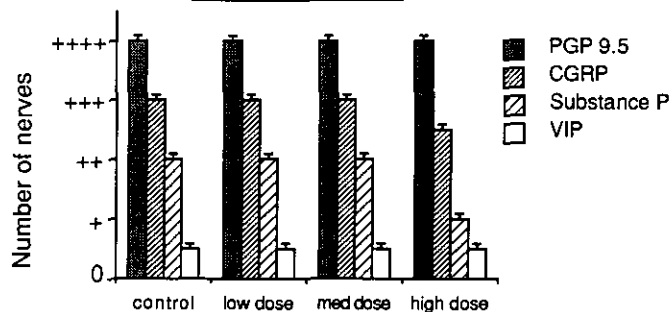


FIGURE 3. Numbers of nerve fibers immunoreactive for PGP 9.5 and neuropeptides in lung, as in Figure 1.

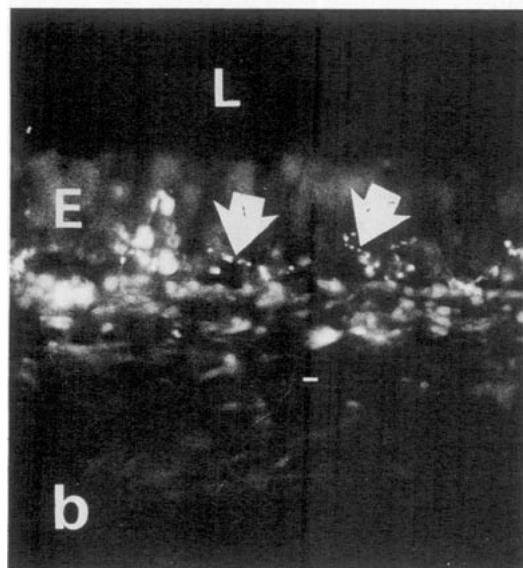
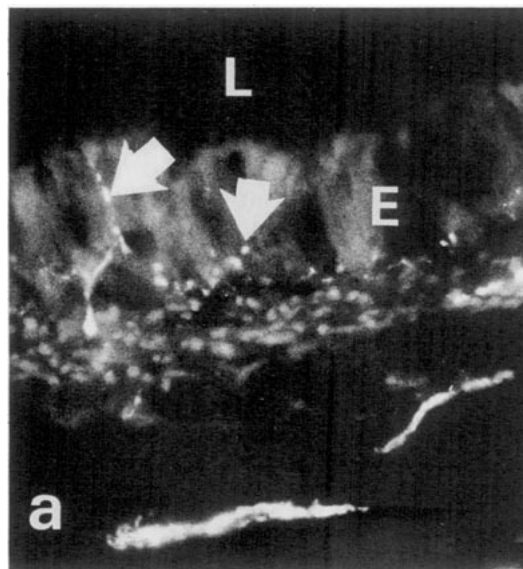


FIGURE 4. Tracheal section immunostained with antiserum to the general neural antigen PGP 9.5. Immunoreactive nerve fibers (arrows) are seen within and below the epithelium (E) of (a) control and (b) high-dose acrolein-treated rats. L, lumen. (a) $\times 600$, (b) $\times 320$.

epithelium, mucosa, smooth muscle and blood vessels of the trachea (Fig. 4), and around airways and blood vessels in the lung (Fig. 5). Scattered fibers were also seen in the lung parenchyma and endocrine cells in the airway epithelium.

CGRP and Substance P

These two neuropeptides are considered together because they had similar distributions and responses to acrolein. Immunoreactive fibers (Figs. 6–8) were seen in and below airway epithelium, in smooth muscle bundles and surrounding blood vessels, and in a proportion of the fibers in nerve bundles in the tracheal adventitia. The number of CGRP-immunoreactive fibers was somewhat greater than those that have substance P staining. The acrolein-treated animals showed a progressive dose-dependent decrease in the number of nerves immunoreactive for either peptide but most markedly substance P (Figs. 1–3, 6–8), and the effect spread further down the respiratory tract with increasing doses. Thus, the low dose ($Ct = 510 \text{ mg}\cdot\text{min}/\text{m}^3$) caused little change in CGRP-immunoreactive nerves with a noticeable decrease in those immunoreactive for substance P in the trachea (Fig. 7), particularly the fibers near the epithelium. The moderate dose ($Ct = 1858 \text{ mg}\cdot\text{min}/\text{m}^3$) caused a greater depletion of CGRP and substance P immunoreactivity and only a few fibers were stainable in the high dose ($Ct = 5693 \text{ mg}\cdot\text{min}/\text{m}^3$) group (Fig. 7). In the lung, changes were seen only in animals receiving the high dose of acrolein, and the depletion was more markedly seen in the substance P-immunoreactive fibers (Figs. 6 and 8). No obvious changes were detected in numbers of epithelial endocrine cells immunoreactive for CGRP.

VIP

Immunoreactive nerve fibers were seen principally in vascular and airway smooth muscle and around glands in the trachea. No change was seen in their numbers in the acrolein-treated animals compared with controls (data not shown).

Discussion

This study has shown that acute treatment with acrolein results in a decrease in the number of nerves immunoreactive for regulatory peptides. Also, it shows that this response is dose related.

Acrolein is a sensory irritant compound, and it is therefore relevant that the two peptides showing changes are substance P and CGRP; these are the two main neuropeptides in the sensory innervation of rodent respiratory tract (24,25). This suggests that the acute exposure to acrolein affects only the sensory nerves and not the autonomic fibers, at least not those containing VIP (mainly postganglionic parasympathetic). It also confirms that acrolein is a sensory irritant. No obvious changes in CGRP-immunoreactive endocrine cells were seen, but a full quantitative study was not undertaken.



FIGURE 5. PGP 9.5 immunoreactive nerve fibers surrounding bronchioles (L, lumen) in the lung of (a) and control (b) high-dose acrolein-treated rats. No change in the number of nerves is seen in the treated animals. $\times 160$.

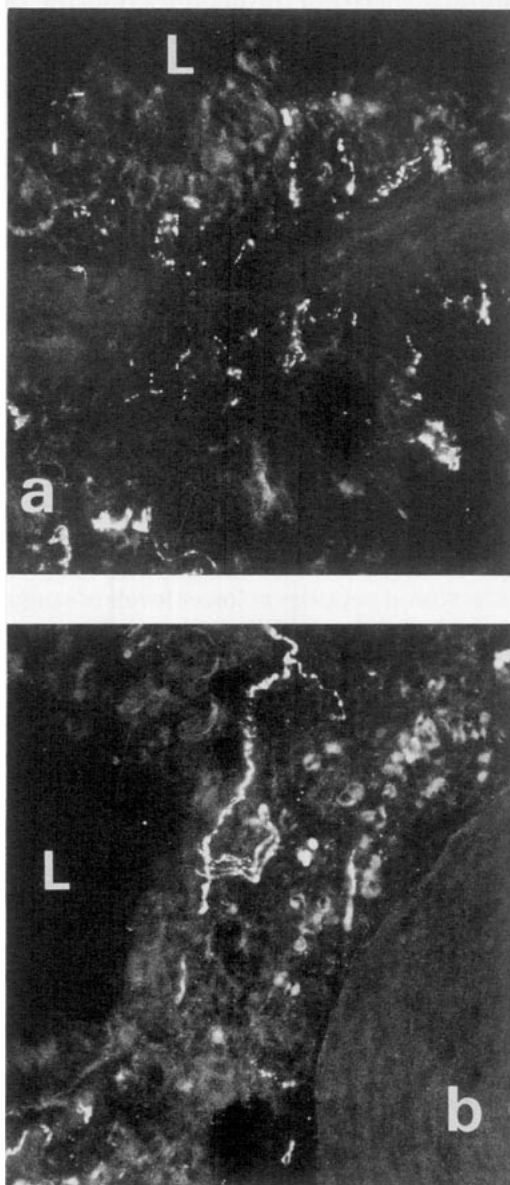


FIGURE 6. CGRP-immunoreactive nerve fibers around a bronchus (L, lumen) in lung of (a) high-dose acrolein-treated and (b) control rats. There is only a slight decrease in nerve fibers stained in the treated group; compare with substance P in lung (Fig. 8). $\times 320$.

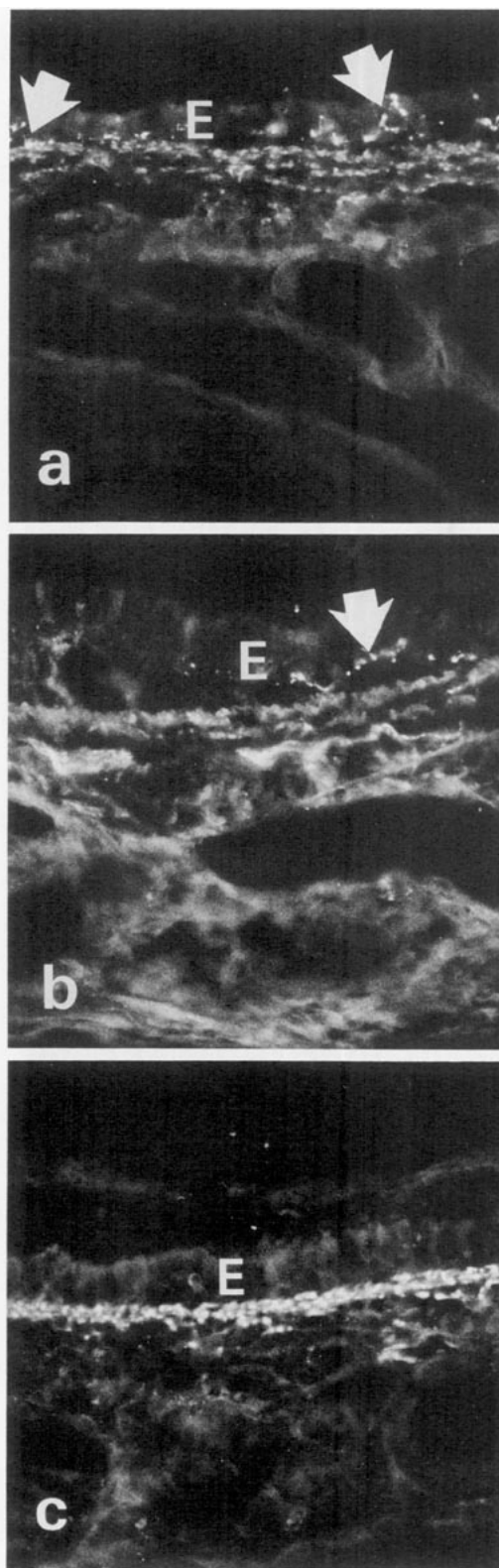


FIGURE 7. Substance P-immunoreactive nerve fibers in rat trachea. The number of nerves (arrows) in epithelium (E) decrease from the control animals (a), to the low dose acrolein treatment (b), and are almost absent from the high-dose treated animals (c). The diffuse staining below the epithelium in (c) is due to autofluorescence of collagen fibers. $\times 320$.

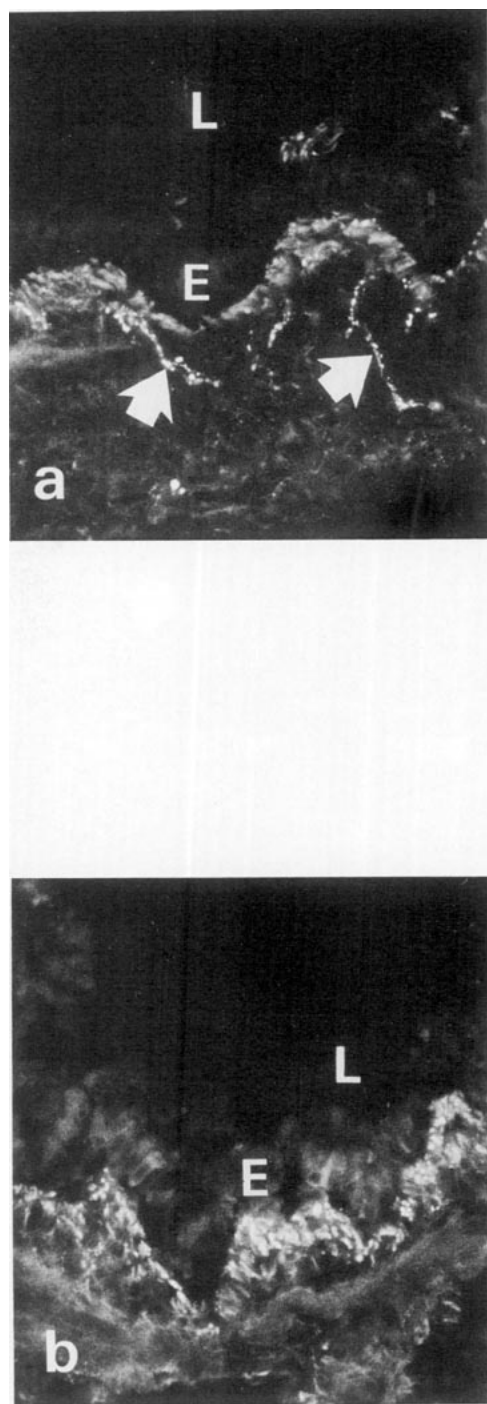


FIGURE 8. Substance P-immunoreactive fibers (arrows) around bronchioles (L) lumen, (E) epithelium in lung, showing a reduction in numbers in high dose acrolein-treated rats (b) compared with controls (a). Note that the diffuse staining below the epithelium in (a) and (b) is due to autofluorescence of collagen fibers. $\times 320$.

It is, therefore, possible that although these cells may be sensory (5), they do not respond to acrolein.

Because of the brief time between initial exposure to acrolein and fixation of tissues, it is unlikely that there was any major structural damage to the nerves, although acrolein is known to cause morphological changes in nerves in the olfactory epithelium of chronically exposed animals, even at low doses (26). The lack of damage is also suggested by the unchanged immunoreactivity for PGP 9.5, a general neural antigen (23) that rapidly disappears following a nerve lesion. Therefore, the reduced CGRP and substance P immunoreactivity must be due to release of the peptides from the sensory nerve fibers by antidromic stimulation. That this mechanism can occur has been shown by studies detecting the released peptides following nerve stimulation (14). This has been shown to occur with other sensory irritants such as cigarette smoke both for substance P (13) and for CGRP, both by immunocytochemistry and radioimmunoassay (unpublished observation).

The apparent greater sensitivity of substance P-immunoreactive nerves is interesting. Substance P and CGRP are known to be co-localized in some sensory nerves (12,27), but in the rat there are more CGRP- than substance P-immunoreactive nerve cell bodies in ganglia innervating the respiratory tract (25). Thus, it is possible that some sensory nerves contain CGRP alone. Where there is co-localization, the peptides are both found in the same secretory granule (27) and therefore must be co-released, which permits CGRP to potentiate the actions of substance P (28). It is possible, therefore, that this type of nerve is more sensitive to acrolein and releases its stored peptides at lower levels of stimulation than those containing CGRP alone, resulting in an apparently greater decrease of immunoreactive substance P nerves than CGRP nerves. Whatever is the case, substance P appears to be a more sensitive indicator of neural response to sensory irritants than is CGRP.

This conclusion is amplified by the observation of a decrease in substance P immunoreactivity in the lung at the high dose level of acrolein, whereas CGRP was little affected. The effects of sensory irritation were less marked in the lung than the trachea, probably because of the high aqueous solubility of acrolein resulting in absorption of most of the vapor in the upper respiratory tract (29). It is therefore probable that had the nasal mucosa been studied here, decreases in sensory peptides would have been detected at lower acrolein concentrations than in the trachea.

The concentrations of acrolein used in this study are high (22, 81, and 249 ppm), compared with the RD_{50} concentration of 1.7 ppm (26). It would be interesting to determine whether the use of lower concentrations for a longer time to give equivalent doses would have similar effects on peptidergic nerves. In rats exposed to cigarette smoke, the effects on neuropeptides of a single exposure are reduced if the animals have been acclimatized to smoke (unpublished observations).

In conclusion, this study has demonstrated that acute

acrolein exposure causes a marked, dose-related depletion of sensory neuropeptides in the rat lower respiratory tract. Evidence suggests that this is due to release of CGRP and substance P from nerve terminals; these released peptides could be involved in the response to acrolein by causing vasodilatation and bronchoconstriction. Thus, it is proposed that investigation of changes in sensory neuropeptides in trachea may be a sensitive indicator of the response to sensory irritants.

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